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USE OF SYNTHETIC AND RECOMBINANT PEPTIDES IN THE STUDY OF HOST-PARASITE INTERACTIONS IN THE MALARIAS

G. H. CAMPBELL,* S. B. ALEY,† W. R. BALLOU,‡ T. HALL,‡
W. T. HOCKMEYER,‡ S. L. HOFFMAN,‡§ M. R. HOLLINGDALE,†
R. J. HOWARD,¶ J. A. LYON,‡ E. H. NARDIN,** R. S. NUSSENZWEIG,**
V. NUSSENZWEIG,** V. C. W. TSANG,* J. L. WEBER,‡ T. E. WELLEMS,¶
J. F. YOUNG,†† AND F. ZAVALA**

*Malaria Branch and Parasitic Diseases Branch, Division of Parasitic
Diseases, Center for Infectious Diseases, Centers for Disease Control, Atlanta, Georgia,
†Malaria Department, Biomedical Research Institute, Rockville, Maryland,
‡Department of Immunology, Division of Communicable Diseases and Immunology,
Walter Reed Army Institute of Research, Washington, DC, §Malaria Branch,
Infectious Diseases Department, Naval Medical Research Institute, Bethesda, Maryland,
¶Laboratory of Parasitic Diseases, National Institute of Allergy and Infectious Diseases,
National Institutes of Health, Bethesda, Maryland, **Department of Medical and Molecular
Parasitology and Department of Pathology, New York University, New York, New York, and
††Molecular Genetics, Smith, Kline and French Laboratories, Swedeland, Pennsylvania

INTRODUCTION*

Recombinant and synthetic peptide technology has allowed new approaches to the study of parasitic diseases. A major benefit of these advances is the ease with which entire genes can be isolated and sequenced at the nucleotide level. This information can help answer questions on genomic organization and evolution. Moreover, we can deduce the entire amino acid sequence of a particular protein without having sufficient protein to visualize on an acrylamide gel. The challenge of translating the nucleotide sequence into a biologically relevant and functional protein remains.

We intend to focus attention on a particular way to accomplish that translation, i.e., the use of polypeptides derived from the deduced sequence to probe or model a function or property of the protein. It is often easier to obtain large amounts of relatively pure recombinant or synthetic polypeptides than of the original protein. Caveats about the use of such peptides cannot be overstated. Proteins are not simply linear entities but often have essential tertiary and quaternary elements that cannot be duplicated by short peptides. The region selected may be in-

ternal in the final protein or processed or modified at an early stage of synthesis.

Despite these pitfalls, a substantial insight has been achieved in studies with recombinant and synthetic peptides. Ongoing work in seven laboratories concerned is summarized.
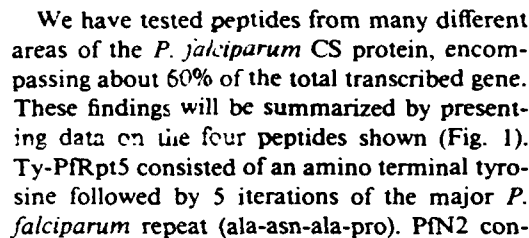
USE OF SYNTHETIC PEPTIDES TO CHARACTERIZE SPOROZOITE-HEPATIC RECEPTOR INTERACTIONS*

A major interest in our laboratory has been the mechanism by which falciparum sporozoites invade the host hepatocytes. Immunization trials in a variety of animal systems have implicated the *Plasmodium falciparum* circumsporozoite (CS) protein as a potential receptor-specific recognition protein.[1-5] This protein is the subject of a great deal of research, some of which will be presented. Because of the difficulties in obtaining and handling this native protein, molecular constructs and synthetic peptides of a deduced sequence are essential tools in these studies.
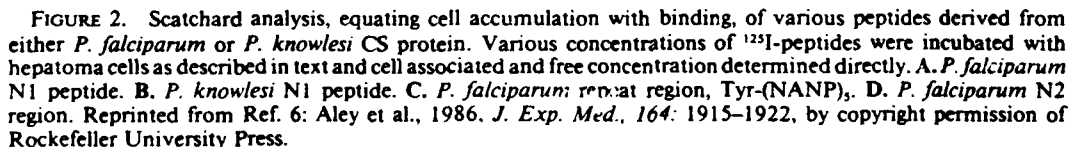
Our studies[6] have emphasized the use of synthetic peptides based on the sequence of Dame et al.[7] The deduced peptide sequence shared several features with CS proteins from other species,[8,9] with all such proteins possessing a central region of tandem repeats that accounted for most of the immune reactivity of the whole pro-

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* A symposium to consider recombinant and synthetic peptide technology in the study of the malarias was held on 8 December 1986 at the 35th Annual Meeting of the American Society of Tropical Medicine and Hygiene. Chairpersons were S. B. Aley and G. H. Campbell.

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* S. B. Aley and M. R. Hollingdale

Peptides used for HepG2 Binding

P. falciparum CS Sequence

FIGURE 1. Summary of synthetic peptides used and their relationship to deduced sequences of CS protein of P. falciparum. Box encloses amino acids conserved between the two sequences.

tein. In addition, Dame et al. identified two regions, one on either side of the repeat region, where the peptide sequences of P. knowlesi and P. falciparum were highly conserved, possibly indicating involvement in some essential function.[7]

We have tested peptides from many different areas of the P. falciparum CS protein, encompassing about 60% of the total transcribed gene. These findings will be summarized by presenting data on the four peptides shown (Fig. 1). Ty-PfRpt5 consisted of an amino terminal tyrosine followed by 5 iterations of the major P. falciparum repeat (ala-asn-ala-pro). PfN2 con-

sisted of the 17 amino acids immediately preceding the repeat region, including all of region I. PfN1 consisted of 11 amino acids immediately preceding the repeat region and including 4 of the 5 most conserved amino acids of region I. PkN1 consisted of 10 amino acids from the sequence of the P. knowlesi CS gene[9] which closely correlated with our N1 region of P. falciparum (Fig. 1). All peptides were synthesized by J. Tam of Rockefeller University and derivatized so that they could be radiolabeled with [125]I.[6]

To test for binding of peptide to target cells, we incubated various concentrations of radiolabeled peptide with HepG2-A16 cells under conditions where invasion by P. falciparum sporozoites can occur, and plotted the results as for a Scatchard analysis, equating cell association with binding (Fig. 2). N1 peptides from either P. knowlesi or P. falciparum reacted in a saturable manner, as indicated by the negative slope of the curve, demonstrating a specific association with target cells. In contrast, both repeat peptide and PfN2 had slopes of essentially zero, indicating a nonspecific or background interaction only. Although the binding of repeat region peptide is

FIGURE 2. Scatchard analysis, equating cell accumulation with binding, of various peptides derived from either P. falciparum or P. knowlesi CS protein. Various concentrations of ^{125}I-peptides were incubated with hepatoma cells as described in text and cell associated and free concentration determined directly. A. P. falciparum N1 peptide. B. P. knowlesi N1 peptide. C. P. falciparum repeat region, Tyr-(NANP)₅. D. P. falciparum N2 region. Reprinted from Ref. 6: Aley et al., 1986, J. Exp. Med., 164: 1915–1922, by copyright permission of Rockefeller University Press.
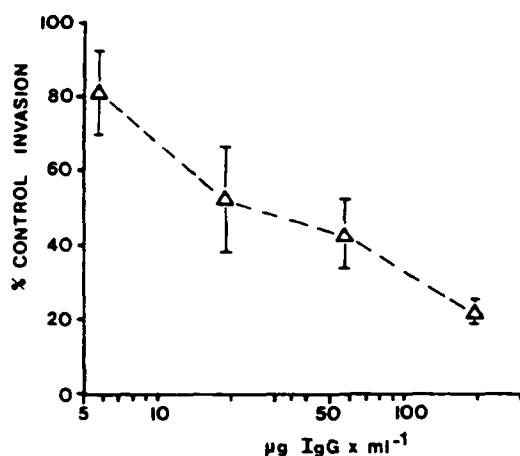
FIGURE 3. Inhibition of sporozoite invasion (ISI) by IgG against the PfN1 peptide. *P. falciparum* sporozoites were incubated in the presence of immune globulin, and the resulting invasion rate was calculated as a percentage of invasion in the presence of normal rabbit immunoglobulin. ISI_{50} = 20 μg IgG per ml. Reprinted with permission.[6]

nonspecific, its level is quite high. We have seen this result with a variety of sizes of repeat peptide as well as with recombinant material.[10] PfN2 reaction is more typical of a nonbinding ligand.

If the N1 region is truly involved in sporozoite/ hepatocyte interaction, then antibodies against that region should inhibit invasion. We tested IgG purified from antisera raised against the PfN1 peptide by U. Vagara at New York University in the inhibition of sporozoite invasion (ISI) assay.[3] The immunoglobulin blocked invasion with a 50% inhibition level at about 20 μg IgG per ml (Fig. 3). Thus far, only sera against either the repeat region or against the N1 region can effectively block invasion of HepG2 cells by sporozoites.

Another prediction of the hypothesis that the N1 peptide is reacting in a ligand/receptor interaction is that we can use the radiolabeled peptide as a probe for the hepatocyte receptor. To test this we reacted HepG2-A16 cells with radiolabeled N1 peptide and washed away nonbound protein. We then used the bifunctional cross-linking agent disuccinyl suberimidate (Pierce Chemical Co., Rockford, Illinois) to covalently link the radiolabeled peptide to its putative hepatic cell receptor. Total proteins were solubilized and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Two proteins of relative molecular size
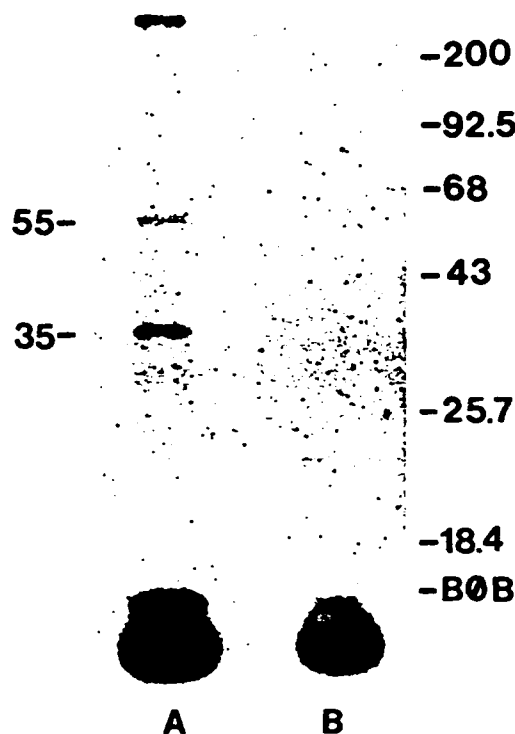
FIGURE 4. Autoradiogram of human liver cell proteins specifically cross-linked to radiolabeled N1 peptide, PkN1. HepG2-A16 cells were incubated with radiolabeled PkN1, washed, and then treated with 200 μg/ml disuccinyl suberimidate (DSS). Unreacted DSS was blocked by addition of glycine. Proteins were solubilized in sodium dodecyl sulfate-sample buffer and separated by SDS-PAGE on 10% acrylamide gels. Proteins covalently linked to the radiolabeled N1 peptide visualized by autoradiography. A. ^{125}I-PkN1. B. ^{125}I-PkN1 + 10 μg/ml unlabeled PkN1. Molecular weight standards are: myosin (200,000), phosphorylase B (92,000), bovine serum albumin (68,000), ovalbumin (43,000), a-chymotrypsinogen (25,700), b-lactoglobulin (18,400), and bromphenol blue. Reprinted with permission.[6]

of 35,000 and 55,000 and presumably of hepatic origin were specifically radiolabeled by this procedure (Fig. 4). Taken together, these findings seem to indicate that the N1 peptide region is directly involved in a specific interaction with HepG2-A16 cells and could be part of the mechanism by which *P. falciparum* sporozoites invade these cells. The role of the repeat region in the invasion process is less obvious. Clearly, antibodies against the repeats can block invasion, but our results argue against a specific receptor/ ligand role for the region. One intriguing possi-

bility is that the *P. falciparum* repeat region may have an amphipathic character that allows it to exist at a polar/nonpolar interface as is found in cell membranes. Such a structure would be able to readily partition into a membrane/water interface but in a nonspecific fashion, possibly explaining the high background binding in our assay. This partitioning would have the effect of bringing the "active" N1 region closer to its target, permitting the "specific" interaction to occur. Antibodies against either structure could then inhibit invasion. One consequence of this model is that the constraints on the structure of the repeat region would not be absolute. In other words, the amino acid sequence of the repeat region could readily vary under immune pressure, as long as the overall structure remained amphipathic.

As a final lesson vis-à-vis this symposium, it is worthwhile to take note of the special case of PfN2. This peptide contains the entire sequence of PfN1, but it does not appear to bind specifically to cells nor do antibodies against it inhibit invasion of target cells.[11] Addition of 6 amino acids, in this case highly positively charged, changes dramatically both the physical and immunological characteristics of a peptide, a strong reminder that the context of a peptide sequence can be as important as the sequence itself. Synthetic peptides are a useful, even essential, tool in this type of research, but we must always remember their limitations.

ANTIGENIC ANALYSIS OF THE REPEAT DOMAIN OF THE CS PROTEIN OF *PLASMODIUM VIVAX**

The gene encoding the CS protein of *P. vivax* sporozoites recently has been cloned and its sequence determined. The primary structure of this protein displays a pattern similar to that of the other CS proteins thus far studied. It has a central 171 amino acid long repeat domain, which constitutes approximately half of the total protein. This domain is composed of a 9 amino acid monomer, repeated in tandem 19 times. Two variants of this monomer occur within the same domain. They differ by a single amino acid residue in the fourth position, which can be alanine (DRAAGQPAG or variant A) or aspartic

acid (DRADGQPAG or variant D). In the CS molecule these variants are arranged as follows: D-D-D-A-D-D-D-D-A-A-D-A-D-A-D-A-A-A-A. Therefore all combinations of consecutive nonamers, i.e., DD, AA, and DA, exist in a repeat domain.

In the present study we investigated whether this variation of the repeats might generate epitope diversity. For this purpose we studied the interaction of various monoclonal antibodies (Mabs) and human sera with the different synthetic peptides and with a recombinant *P. vivax* CS protein expressed in yeast. To determine the fine specificity of these antibodies against the *P. vivax* CS protein, we performed competitive binding assays, using synthetic peptides with 9 (A, D) and 18 amino acids (AA, DA, and DD) and the recombinant CS vivax protein, which expresses the entire repeat domain plus short stretches of the N- and C-terminal domains.

The results indicate that the A monomer only inhibited the binding of Mab 2F2, whereas the D monomer inhibited the binding of 2F2 and 3 additional Mabs (4E8, 1H8, and 3D10). Using the AA dimer we found that it also inhibited only the Mab 2F2. In contrast, the DA dimer, which differs from AA by a single amino acid residue, strongly inhibited all 6 Mabs. The DD dimer also inhibited the binding of all 6 Mabs, although less efficiently.

The distinct epitopes clearly are recognized by these Mabs. This diversity of epitopes may have originated by the substitution of an alanine for an aspartic acid in the fourth position of some of the repeats. It appears that the CS protein of *P. vivax* contains at least two different repeating epitopes that may be partially overlapping.

The results obtained with human sera further corroborate the different epitope specificity of antibodies directed against the *P. vivax* CS protein. A noteworthy finding was that the reactivity of some of these sera with the recombinant CS vivax protein could not be inhibited by the synthetic peptides corresponding to the repeat domain. This lack of inhibitory capacity may be due to the presence of antibodies directed against epitopes localized outside of the repeat domain and/or the presence of antibodies against conformational determinants, which fail to be represented by the synthetic peptides.

These results suggest that the recombinant CS protein of *P. vivax* reflects better than the synthetic peptides the antigenic characteristics of the

* F. Zavala

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TABLE 1

Antibody response to R32LR in mice immunized with R32tet₃₂ and R32LR

Immunogen	Adjuvant	ELISA absorbance (414 nm)*
R32tet₃₂	None	2.75
R32LR	None	0.02
R32tet₃₂	CFA/IFA	2.87
R32LR	CFA/IFA	1.53

* Results are mean absorbance of triplicate sera at a serum dilution of 1:100.

native protein. It may therefore be advantageous to use the recombinant protein in epidemiological studies aimed at detecting antibodies against *P. vivax* sporozoites. The findings are also relevant for vaccine development, although immunogenicity and protective immunity will be the most important criteria.

IMMUNOGENICITY OF A RECOMBINANT DNA PLASMODIUM FALCIPARUM SPOROZOITE VACCINE IN MICE AND HUMANS*

The CS protein of *P. falciparum* is composed of 412 amino acids, 40% of which are included in the 41 tandemly repeated tetrapeptides (37 NANP and 4 NVDP) of the central immunodominant region of this protein.[7] A recombinant DNA construct, R32tet₃₂, derived from the CS protein of *P. falciparum* was shown to be highly immunogenic in laboratory animals.[4] The results of additional preclinical studies which led to the choice of R32tet₃₂ as the first subunit malaria vaccine[12] and the results of phase 1 studies of the safety and immunogenicity of R32tet₃₂ in humans[13] are summarized.

R32tet₃₂ consists of 32 tetrapeptide repeats (R32) from the CS protein, ([NANP]₁₅NVDP)₂, fused to 32 amino acids (tet₃₂) derived from the tetracycline resistance gene of the plasmid. Although preclinical studies indicated that R32tet₃₂ was safe and immunogenic in laboratory animals,[4] two potential problems exist with R32tet₃₂. First, if the tet₃₂ portion of the fusion protein acted as a carrier protein for T lymphocyte recognition and no T cell epitopes were present on the R32 portion of the construct, then natural exposure to sporozoites would not boost the im-

* S. L. Hoffman, W. R. Ballou, J. F. Young, and W. T. Hockmeyer

mune response elicited by immunization with R32tet₃₂. Secondly, a vaccine made without the tet₃₂ portion of the fusion protein would offer theoretical advantages if it was immunogenic, since tet₃₂ is extraneous in regard to the specific desired immune response.

Therefore, we produced another construct (R32LR), which was identical to R32tet₃₂, except that it included only the first two amino acids from the tet₃₂ fusion protein (leucine and arginine). When C57BL/6 mice were immunized with R32tet₃₂ or R32LR in phosphate buffered saline (PBS), only the mice immunized with R32tet₃₂ made antibodies which recognized R32LR by enzyme-linked immunosorbent assay (ELISA) (Table 1). To determine if the absence of an antibody response after immunization with R32LR was attributable to the lack of a T cell epitope on this construct, we immunized mice with the two constructs emulsified in complete Freund's adjuvant (CFA). Mice immunized with R32tet₃₂ or R32LR emulsified in CFA produced antibodies that reacted with R32LR (Table 1), indicating the presence of a T cell epitope on the CS protein-derived portion of the molecule. These findings were corroborated by studies of T cell proliferation, which showed that lymph node cells (LNCs) from C57BL/6 mice immunized with R32tet₃₂ or R32LR in CFA proliferated after stimulation with R32tet₃₂ or R32LR. A proliferative response was seen when LNCs from mice immunized with R32tet₃₂ were stimulated with the synthetic peptide PNANPNA, indicating that the T cell epitope was ≤7 amino acids. Both antibody and T cell proliferative responses indicated that R32tet₃₂ was more immunogenic in C57BL/6 mice than was R32LR (Tables 1, 2). The superior immunogenicity of R32tet₃₂ might be partially explained by the presence of a T cell epitope on the tet₃₂ tail. Mice were immunized with R32tet₃₂, and their LNCs were stimulated with R32tet₃₂ or R1V20tet₃₂, a *P. vivax* CS protein construct that shared amino acid sequence homology with R32tet₃₂ only in the tet₃₂ tail and was not recognized by Mabs against *P. falciparum* CS protein. Both constructs stimulated proliferation of LNCs primed with R32tet₃₂, indicating the presence of a T cell epitope on the tet₃₂ tail.

An additional experiment showed that mice immunized with R32tet₃₂ produced a secondary antibody response after intravenous injection of *P. falciparum* sporozoites (Table 3). This sug-

TABLE 2

Proliferative response of lymph node cells from mice immunized with R32tet₁₂ aad R32LR

Immunogen	In vitro stimulation	Stimulation index*
R32tet₁₂	R32tet₁₂	18.6
R32tet₃₂	R32LR	12.1
R32LR	R32tet₃₂	11.4
R32LR	R32LR	6.1

* Results are expressed as the ratio of counts per minute of stimulated and :: *mulated cells (stimulation index) at the optimal in vitro antigen concentration for that experiment (20–60 µg ml).

TABLE 3

Antibody response to R32tet₁₂ in mice can be boosted by secondary immunization with sporozoites

Day 0*	Day 21	ELISA absorbance (414 nm)
R32tet₃₂	Sporozoites	2.99
R32tet₃₂	None	0.18
None	Sporozoites	1.56

* Mice received an intramuscular injection of R32tet₁₂ mixed with aluminum hydroxide on day 0 and an intravenous injection of *P. falciparum* sporozoites on day 21. Control mice received R32tet₁₂ on day 0 or sporozoites on day 21. Serum specimens taken on day 28 were tested for antibody to R32tet₁₂. Results are mean absorbance values of triplicate sera at a serum dilution of 1:400.

gested that boosting of antibody might occur after natural exposure to sporozoites. These studies indicated that R32tet₃₂ was the best available vaccine candidate. It was highly immunogenic in C57BL/6 mice and in rabbits, included a T cell epitope within the R32 or CS protein-derived portion of the fusion protein, and elicited an antibody response that could be boosted by exposure to sporozoites.

R32tet₃₂ adsorbed to aluminum hydroxide, falciparum sporozoite vaccine-1 (FSV-1), was tested for safety and immunogenicity in 15 human volunteers. The volunteers were divided into 5 groups with each group receiving a different dose of FSV-1 (10, 30, 100, 300, or 800 µg). The same dose of vaccine was administered 3 times at 4-week intervals.

The vaccine was generally well tolerated. Mild pain associated with the injection occurred in 7/9 individuals who received ≥ 100 µg. Tenderness at the immunization site was detected in 8/15, including all individuals who received 300 or 800 µg. One individual developed sneezing and generalized urticaria, without associated hypotension or dyspnea, 5 min after the last 800 µg dose. This resolved spontaneously after 30 min. Only this individual developed IgE antibodies specific for R32tet₃₂. Twelve of 15 volunteers produced antibodies that reacted with R32LR by ELISA. The magnitude of the antibody response increased with increasing doses and never reached a plateau. Antibody levels ranged from <1 µg/ml to 50 µg/ml. Only one individual, who was in the group receiving 800 µg, developed an antibody titer >1:400 by ELISA and indirect fluorescent antibody (IFA) assay. Sera obtained 2 weeks after the third dose of FSV-1 from 9 of 15 volunteers produced a 2+ CS precipitation reaction. When compared to each individual's preimmunization serum specimen, sera taken 2 weeks after the third dose of

FSV-1 inhibited sporozoite invasion into hepatoma cells from 12% to 85% (median 50%). Peripheral blood mononuclear cells from 11/13 volunteers studied proliferated in response to stimulation with R32tet₃₂. Four weeks after the first dose the mean stimulation index of the 13 volunteers was 13.0 at the optimal R32tet₃₂ concentration of 15 µg/ml. Prior to immunization the mean stimulation index in the same 13 individuals was 2.5. The proliferative response did not change after booster doses, nor was it correlated with the dose of vaccine received or the antibody response.

The magnitude of the human antibody responses was lower than expected based on the preclinical animal studies. The results are encouraging, however, in that 100% of human volunteers made an immunologic response to the vaccine as measured by antibody production or lymphocyte proliferation, and the concentration of antibody increased with increasing doses of the vaccine and never reached a plateau. Studies are underway to improve the immunogenicity of CS protein subunit vaccines in humans and to determine if the immunity elicited by immunization with FSV-1 will protect against sporozoite challenge.

T AND B CELL RESPONSES IN MICE IMMUNIZED WITH RECOMBINANT *PLASMODIUM VIVAX* CIRCUMSPOROZOITE PROTEIN*

The gene encoding the CS protein of *P. vivax* recently has been cloned and the immunodominant B cell epitope characterized.[14,15] The pro-

* E. H. Nardin, R. S. Nussenzweig, and V. Nussenzweig

tein consists of 373 amino acids with a central region of 19 tandem repeats of a nonapeptide. Synthetic dimers of the nonapeptide bind Mabs directed to the CS protein of *P. vivax* and inhibit the interaction of antibody with the native protein obtained from sporozoite extract.[14] Yeast cells have been transformed with plasmids containing DNA coding for 234 amino acids of the vivax CS protein.[16] The expressed recombinant protein (rCS) contains the entire repeat region plus 15 amino acids preceding the repeats and 48 amino acids following the repeats on the C-terminal side of the protein.

Outbred Swiss-Webster (SW) mice immunized with purified recombinant vivax CS protein adsorbed to alum developed anti-CS antibodies.[16] Pooled sera obtained after the second booster gave titers of 1:1,000 when tested against *P. vivax* sporozoites by IFA assay. The sera of the immunized mice also effectively blocked invasion of a human hepatoma cell line by viable *P. vivax* sporozoites.

The studies presented describe the in vitro responses of cells obtained from these immunized outbred SW mice as well as the in vivo responses of inbred strains of mice immunized with the *P. vivax* rCS protein.

Spleen cells obtained from the rCS/alum-immunized SW mice were cultured in vitro with various concentrations of recombinant *P. vivax* protein, synthetic peptides representing the immunodominant nonapeptide repeat sequence, or parasite extracts obtained by sonication of salivary gland sporozoites. Immune responses were measured by lymphocyte proliferation and the production of antisporozoite antibodies in vitro. Specific antibodies in the cell supernatants were detected by IFA assay using *P. vivax* sporozoites as antigen or by ELISA using rCS-coated plates.

Immune spleen cells from rCS mice proliferated when challenged in vitro with rCS at a concentration of 50 μg/ml. Antisporozoite antibodies could be detected by both IFA and ELISA in the supernatants of immune cells challenged with the rCS protein. Similarly, the native CS antigen, contained in the sonicated extract of *P. vivax* sporozoites, stimulated lymphocyte proliferation and induced production of antisporozoite antibodies in vitro. Dimers of the repeat nonapeptide failed to induce proliferation or antibody production.

Proliferation did not directly correlate with the production of antisporozoite antibodies in vitro. Anti-*P. vivax* sporozoite antibodies could be detected in the absence of high levels of proliferation. In addition, high levels of nonspecific proliferation were frequently observed when using sporozoite extracts due to T cell mitogens found in the mosquito salivary gland debris. In spite of the presence of nonspecific proliferation, however, antisporozoite antibodies were not detected in the supernatants of control wells, i.e., immune spleen cells challenged with extracts of normal salivary glands or heterologous species of sporozoites.

The generation of antisporozoite antibody in vitro is T cell dependent. Immune cells depleted of T cells by reaction with anti-Thy 1 plus complement did not produce detectable levels of antisporozoite antibodies when incubated with sporozoite extracts in vitro. Therefore, in contrast to proliferation, detection of antisporozoite antibodies in the culture supernatants of immune spleen cells reflects a specific immune response in the in vitro assay.

To identify the T cell epitope on the rCS vivax protein, we immunized inbred mice with rCS. Recent experiments have shown that the murine response to the repeat region of the *P. falciparum* CS protein is genetically restricted.[17, 18] When congenic strains on the B10 background were immunized with rCS adsorbed to alum, only the sera of B10.BR (H-2k haplotype) and E10.A (H-2a haplotype) mice gave positive IFA assay reactions to *P. vivax* sporozoites. The immune response to vivax rCS protein was restricted by the k allele of I-A, as shown by the ability of mice of B10.A and the recombinant strain B10.A (4R), but not B10.A (5R), to respond to rCS.

In summary, T cells of outbred SW mice immunized with vivax rCS protein recognize native sporozoite antigen in parasite extracts and respond as helper cells in the antisporozoite antibody response in vitro. T cells of immunized individuals would therefore be expected to respond to challenge by the bite of malaria-infected mosquitoes under natural conditions. It remains to be determined whether humans, the natural host of *P. vivax* parasites, would respond to immunization with rCS vivax protein similar to the "unnatural" mouse host and develop an immunological response similar to the outbred SW mice or, alternatively, be genetically restricted in their response as are the inbred congenic strains.

USE OF SYNTHETIC PEPTIDES IN
SEROLOGIC ASSAYS*

Traditionally, serologic assays have been used to determine a person's exposure to the malaria parasite, P. falciparum, by determining the presence of antibody to that organism in the sera of the individual. The antibodies detected are specific to a multitude of independent epitopes on enumerable parasite proteins. Measurement of antibody specific to individual parasite proteins is difficult because of the crude preparations of parasite proteins that have been used in serologic assays. Crude antigen mixtures also may react with antibodies elicited by parasites of more than one species or genus. Thus, the presence of antibodies as determined by such assays can only be used as a measure of some undefined previous exposure of the host to the malaria parasite. Furthermore, host- or culture-derived parasite materials are difficult to obtain in sufficient quantity and purity necessary for immunoassays. The advent of recombinant and synthetic peptide technology has led to the capability to produce large quantities of peptides from specific proteins of the malaria parasite. This permits dissection of the antibody response of humans and animals with regard to specific individual proteins. The potential of recombinant and synthetic peptide technology in mass production of specific parasite proteins may allow new types of information to be gained from large epidemiological studies.

Using these purified products from specific parasite proteins, several questions can be asked regarding the host immune response to the parasite during natural infection. The serologic response of a population to a particular parasite protein as opposed to the responses measured using a crude mixture of antigens can be evaluated. A follow-up question is the age at which individuals in a population develop antibody to a particular protein in relation to intensity or duration of exposure to malaria parasites. Of importance are the correlation of an antibody response to a particular protein and ability of a host to be protected against initial or severe infection.

Reliable answers to many of these questions will require immunoassays that are quantitative,

* G. H. Campbell and V. C. W. Tsang

reproducible, and adaptable for use with large numbers of sera. Synthetic peptides derived from known amino acid sequences of P. falciparum proteins were used in an ELISA as previously developed for species-specific determination of antibody to Schistosoma sp. parasites.[19] The assay is kinetic based; that is, the amount of activity of antibody-bound enzyme is determined by the initial rate of the substrate-enzyme reaction. The requirement for such an assay is that all reagents are present in excess except the one being quantitated, in this case, antibody to a particular peptide. In addition to being highly quantitative and reproducible, the procedure is easily adaptable to large scale application.

The physical configuration of the assay described here to measure antibody to malaria parasite peptides is based on the Falcon assay screening test (FAST, Becton Dickinson Labware, Oxnard, California). This consists of a polystyrene microtiter plate and a lid that contains strips of beads on sticks, one bead per well of the microtiter plate. Various peptides are placed in microtiter wells and coated onto beads by placing the lid with knobs into the plate wells for 2 hr at room temperature while shaking on a mini-orbital shaker. After sensitization of the knobs with the peptides, plate lids are washed by spraying with PBS containing 0.5% Tween 20, and then rinsed with deionized water. The spraying also insures that the coated knobs are thoroughly washed between steps of the assay. Peptide-coated plate lids are then air dried and stored over desiccant at room temperature. The assay is performed by sequentially submerging the peptide-coated knobs in microtiter plates containing the human sera to be tested for antibody (test antisera), goat anti-human IgG (second antibody), and the 3,3',5,5'-tetramethylbenzidine (TMB) (Miles Laboratories, Naperville, Illinois) and H_2O_2 substrate system. Plate lids are sprayed and rinsed between each 5 min shaking incubation step of the assay. After the final 5 min incubation in the substrate-containing plate, the lid is removed and discarded. The absorbance values of the wells are then determined at a wavelength of 620 nm using an ELISA plate reader.

The synthetic peptides used in this study were produced using the amino acid sequences of two proteins of P. falciparum, the CS protein[7] and the ring-infected erythrocyte surface antigen
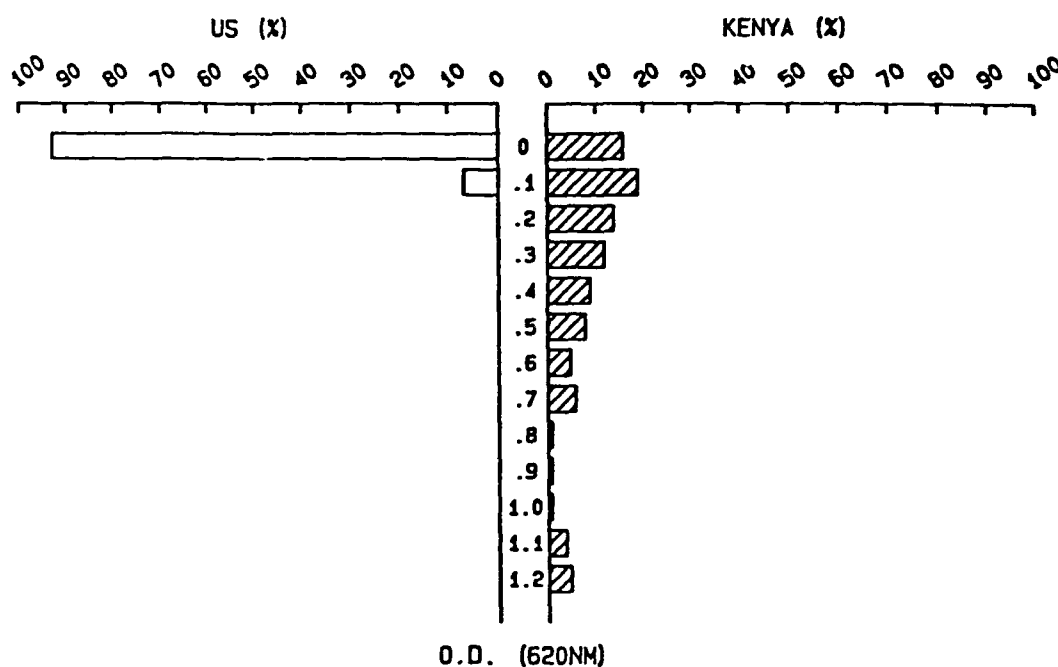
FIGURE 5. Frequency distribution of antibody reactivity of 75 U.S. and 80 Kenyan serum specimens against the synthetic peptide (PNAN)₅.

(RESA).[20] All peptides represent naturally existing tandemly repeated amino acid sequences from their respective proteins. The synthetic peptides were: (PNAN)₅ from the CS protein, (EENV)₅ and (EENVEHDA)₄ from the 3' region of the RESA protein, and (DDEHVEEPTVA)₃ from the 5' region of the RESA protein. Antibody reactivities in human sera were determined using each of these peptides.

Two study populations were compared. Serum specimens from 75 healthy U.S. volunteers were obtained from the Centers for Disease Control serum bank. Eighty serum specimens were obtained during a survey conducted in October 1985 in the Kisumu area of Kenya.[21] Figure 5 shows the distribution of absorbance values of U.S. and Kenyan sera when tested for antibody to the sporozoite peptide (PNAN)₅. All of the U.S. sera had absorbance values of <0.15. The 95th percentile ranking of the absorbance values was 0.052. This value can then be used as a cut-off for determining positive reactions. Numerous Kenyan serum specimens reacted above this level (Fig. 5). The same analysis was performed with the U.S. serum specimens and the RESA peptides, (EENV)₅, (EENVEHDA)₄, and (DDEHVEEPTVA)₃. Values greater than 0.114,

0.189, and 0.243 were considered positive for each respective peptide.

The distribution of absorbance values of the Kenyan sera reacting with the RESA peptides is compared to reactivity with the CS peptide in Figure 6. Although reactivity with the CS peptide was more intense in a larger percentage of the sera than with the RESA peptides, strong reactions were observed with some sera to all of the peptides.

The percentage of sera reactive with each of the peptides is shown (Fig. 7) and compared with the 100% reactivity of the Kenyan sera in an IFA assay using P. falciparum parasites. The age distribution of the donors of these sera is shown (Fig. 8). A clear relationship between increasing age and the percentage of sera reactive with a particular peptide is seen with three of the peptides. The reactivity of the younger age groups with the peptide (EENV)₅ may be an anomaly because the absorbance values of the positive sera in the 0-4 and 5-9 age groups were in all cases only slightly above the cut-off value for designation as a positive. The response to the CS peptide appears to develop at an earlier age than responses to the other two RESA peptides.

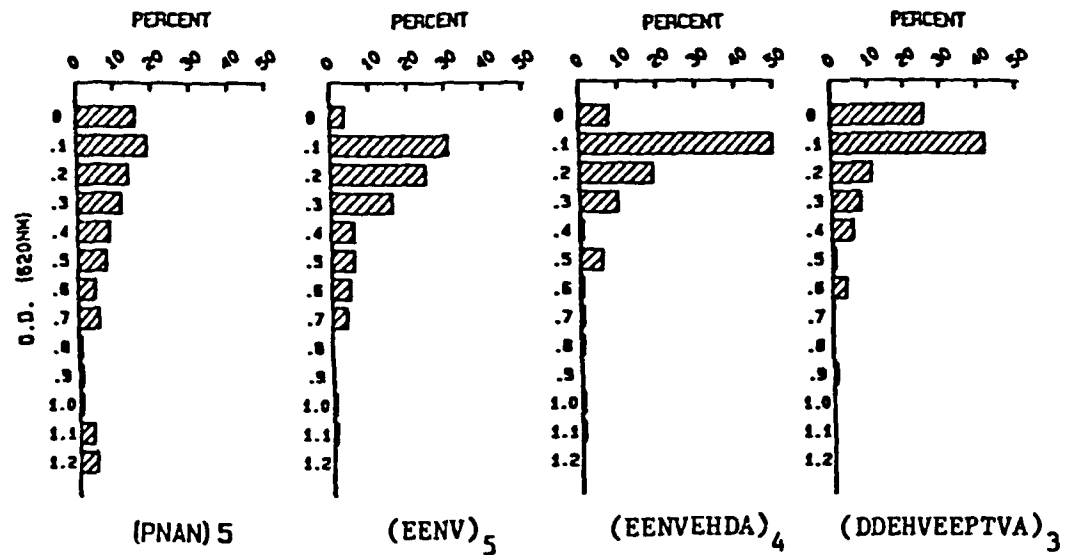The findings show that it is possible to detect

FIGURE 6. Frequency distribution of antibody reactivity of Kenyan serum specimens against synthetic peptides.

antibodies to distinct proteins of *P. falciparum* by using the FAST-ELISA with synthetic peptides as antigens. The differences in reactivity to various peptides of the same parasite (e.g., CS protein vs. RESA protein) in the same population are remarkable. Although the significance is not yet clear regarding such issues as specific immunity, this study does show that a population-based antibody specificity spectrum does exist. Further definition of these differences in antibody reactivity to various synthetic peptides may prove important in understanding the immune response to infection with malaria parasites.

pendently of knobs[23] and is released from intact infected red blood cells into the culture supernatant and body fluids.[24] HRP-III, also designated as SHARP, is a third HRP recently identified by molecular cloning techniques.[25, 26] The gene encoding HRP-III exhibits a close evolutionary relationship to that encoding HRP-II.[26] HRP-III is not phenotypically associated with the development of knobs; the exact location of HRP-III is undetermined.

Several laboratories have described nucleotide sequences for the genes encoding HRP-I, HRP-II, and HRP-III,[25-27] which should aid investigations of the biological properties of these pro-

USE OF SYNTHETIC OLIGOPEPTIDES TO CHARACTERIZE HISTIDINE-RICH PROTEINS OF *PLASMODIUM FALCIPARUM* [*]

P. falciparum produces an unusual family of proteins distinguished by extraordinarily high contents of histidine (histidine-rich proteins or HRP). Properties of 3 such proteins produced by the erythrocyte stage parasite are summarized (Table 4). HRP-I, the knob-associated protein described by Kilejian, is associated with the development of knob-like protrusions involved in attachment between infected erythrocytes and endothelial cells.[22] HRP-II is synthesized inde-
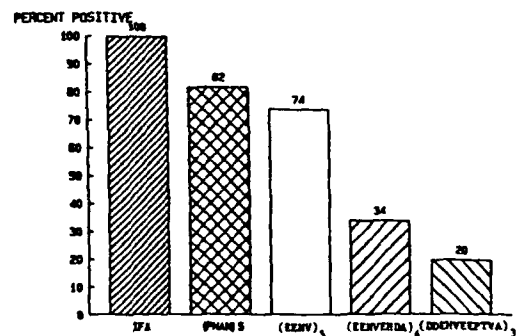

FIGURE 7. Percentage of Kenyan sera positive in indirect fluorescent antibody (IFA) assay and enzyme-linked immunosorbent assay (FAST-ELISA) with synthetic peptides.
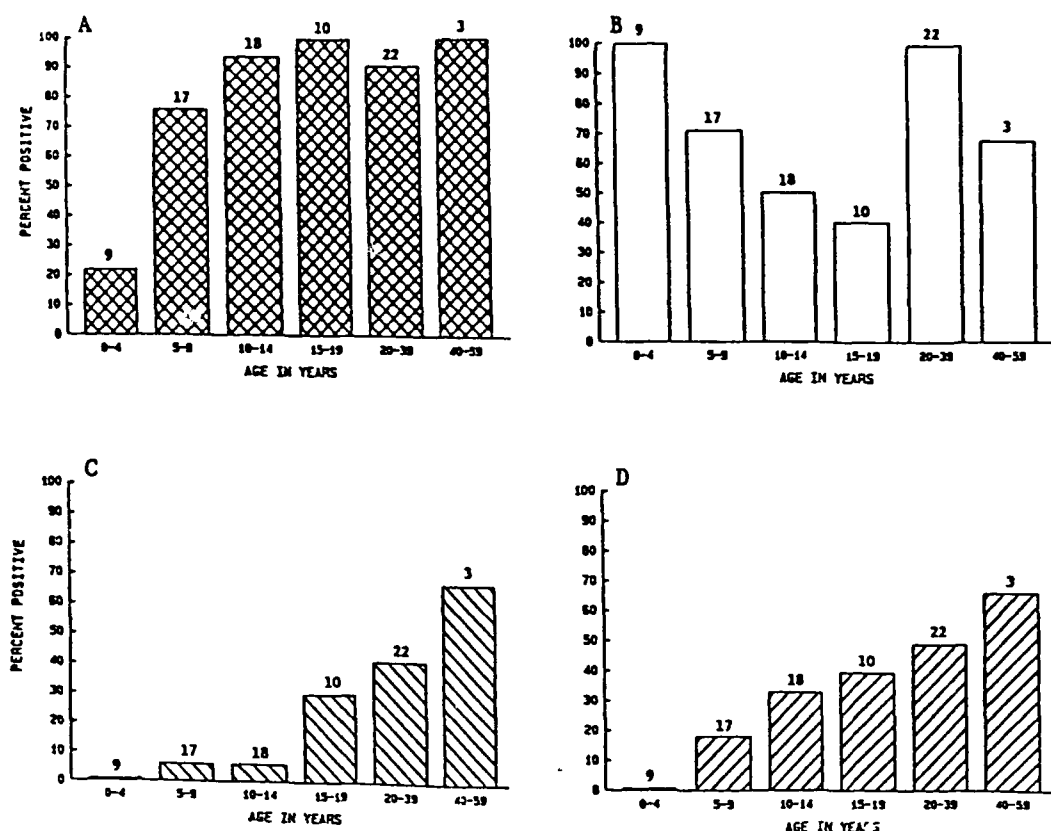
* T. E. Wellems

FIGURE 8. Age distribution of antibody reactivity to synthetic peptides. Numbers above bars represent the number of specimens in each age group. A. (PNAN)₅. B. (EENV)₅. C. (DDEHVEEPTVA)₃. D. (EENVEHDA)₄.

teins. This presentation describes the use of synthetic peptides generated on the basis of nucleotide sequence data to identify and characterize HRP-II and HRP-III.

A schematic illustration of the genomic organization of the HRP-II and HRP-III genes is shown (Fig. 9). Both genes have a similar structure, with an intron dividing an exon encoding a hydrophobic leader from an exon encoding tandem repeats rich in histidine and alanine. Regions flanking the tandem repeats, including untranslated regions, show very high levels of nucleotide sequence homology, implying the genes have arisen by duplication and divergence from a common ancestor.[26] In the repeat regions, the sequences have diverged in a remarkable way. A single, continuous block of tandem repeats occurs in HRP-II, whereas two blocks of repeats separated by a nonrepetitive region of protein are present in HRP-III. The HRP-II repeats

comprise mainly the tripeptides AHH, the hexapeptides AHHAAD, and 3 pentapeptides AHHAA.

In contrast, HRP-III has two blocks of repeats: a 5' repeat domain containing the tripeptide and hexapeptide units AHH and AHHAAN; and a 3' repeat domain containing tandemly repeated pentapeptides, mainly DDAHH and DGAHH. Thus, the blocks of repeats are all related in that they contain common residues of alanine and histidine but otherwise vary in amino acid composition. The predominant hexapeptide unit in HRP-II, AHHAAN, differs from that in HRP-II by a change in the sixth amino acid from an aspartate to an asparagine residue. The 3' block of pentapeptide repeats and the preceding nonrepetitive stretch of protein in HRP-III have no counterpart in HRP-II.

The divergence between the repeat domains of HRP-II and HRP-III has made it possible to

TABLE 4

Histidine-rich proteins of P. falciparum

HRP-I (KA-HRP)

Knob-associated histidine-rich protein.

Production necessary but not sufficient for cytoadherence.

Tightly associated with the cytoskeleton.

M_r by SDS-PAGE 80,000 to 120,000.

HRP-II

Secreted from parasitized red blood cell into extracellular medium.

Not associated with knob phenotype or cytoadherence.

High content of alanine and aspartic acid.

M_r by SDS-PAGE 65,000 to 85,000.

HRP-III (SHARP)

Closely related to, but distinct from, HRP-II.

Not associated with knob phenotype or cytoadherence.

High content of alanine and asparagine.
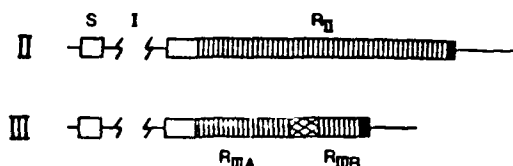
M_r by SDS-PAGE 30,000 to 50,000.

FIGURE 9. Schematic diagram of the organization of the genes encoding HRP-II and HRP-III in clone 7G8 of Brazil *P. falciparum* isolate IMTM22. Coding regions are represented by boxes. Repeat domains are indicated by vertical bars. Both genes comprise an exon encoding a hydrophobic leader (S), an intron (I), and a 3' exon encoding the tandem repeats. The 3' exon of HRP-II contains a long stretch of repeats, predominantly, AHH and AHHAAD. The 3' exon of HRP-III has two blocks of repeats, a 5' block (R_{IIIA}) comprising predominantly the repeats AHH and AHHAAN, and a 3' block (R_{IIIB}) which contains the pentapeptide repeats DDAHH and DGAHH. The nonrepetitive stretch of protein between the two blocks of repeats in HRP-III (cross-hatch) has no counterpart in HRP-II.

generate synthetic peptides and antisera specific to each protein. Results are shown (Fig. 10) of immunoprecipitations with rabbit antisera raised against the peptide AHHAHHAADAHHAAD. derived from the HRP-II repeats, and against the peptide GAHHDDAHHDGAHHD, derived from the HRP-III repeats. The antisera clearly distinguish HRP-II and HRP-III. HRP-II (from the 3D7 clone of *P. falciparum*) has a relative molecular weight of 72,000 by SDS-PAGE (Fig. 10. lane 1), while HRP-III has a relative molecular weight of 48,000 (Fig. 10, lane 2). That these ^{3}H-histidine labeled bands are indeed HRP-II and HRP-III has been confirmed by two observations: 1) the bands are detected in labeling experiments with ^{3}H-alanine but not ^{3}H-isoleucine, as expected from the deduced amino acid sequence;[26] and 2) the bands are absent from parasite isolates in which the genes encoding HRP-II or HRP-III are deleted (T. E. Wellems, personal communication).

Availability of synthetic peptides and antibody probes specific for HRP-II and HRP-III will facilitate investigations into the localization and processing of these proteins within the parasite. Immunoelectron microscopy with rabbit antisera against the HRP-II repeats has confirmed transport of this antigen from the parasite, through the cytoplasm, and across the cell membrane of red blood cells.[24] The means by which

the membranes of the parasite vacuole and the red blood cell are crossed and the mechanism of transport through the red blood cell are not understood.

The use of synthetic peptides should clarify the biological properties and functions of HRP-II and HRP-III. The possible roles of HRP in neutralizing free radicals, interacting with the host immune system, and chelating divalent cations
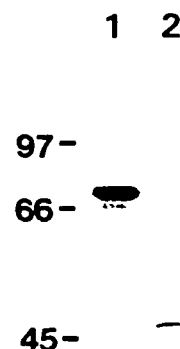

FIGURE 10. Immunoprecipitation of HRP-II and HRP-III from extracts of the 3D7 clone of *P. falciparum*. Parasites were radiolabeled with ^{3}H-histidine, extracted with Triton X-100, and immunoprecipitated as described.[23] Rabbit antisera against the synthetic peptides AHHAHHAADAHHAAD (lane 1) and GAHHDDAHHDGAHHD (lane 2) were raised by coupling the peptides to keyhole limpet hemocyanin and injecting with Freund's complete adjuvant. Molecular weight standards are indicated in kDa.
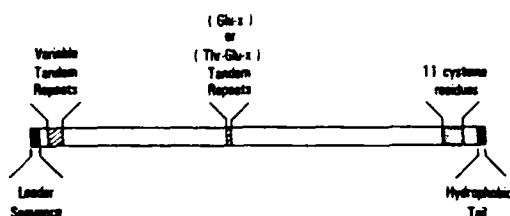
FIGURE 11. Schematic diagram of structural features of the gene encoding gp195.

have been discussed.[28] Investigations using the different peptides may determine whether HRP-II and HRP-III have distinct biological properties arising from their different primary structures, or whether they have similar properties, perhaps deriving from their unusually high histidine content.

THE USE OF SMALL PEPTIDES IN STUDIES ON THE MAJOR POLYMORPHIC SURFACE GLYCOPROTEIN FROM *PLASMODIUM FALCIPARUM* MEROZOITES*

Synthetic and recombinant peptides were used to evaluate antibody responses to a malaria antigen (gp195) which is the precursor to several major antigens found on the surface of *P. falciparum* merozoites. The results illustrate some of the benefits and hazards associated with using peptides to investigate immune responses to antigens. Benefits: antibodies can be raised against regions of the protein that are not normally immunogenic, and monospecific polyclonal antibodies can be produced by immunizing with peptides. Some of the hazards are: some regions of a peptide may be more immunogenic than others; the chemistry of coupling peptides to carriers may influence the production of antibody against the peptide; antibodies raised against a peptide may react in some assay systems but not others; and antibodies raised against a peptide from one protein may cross-react with other proteins.

The strain-variable 195 kDa glycoprotein, gp195, is synthesized by *P. falciparum* trophozoites and schizonts and is processed by proteases into at least 4 lower M, products found on merozoites.[29-37] Products identified include: p83, which in the Camp strain is further processed sequentially into 73 and 67 kDa products; p45;

* J. A. Lyon, T. Hall, and J. L. Weber

gp45, which is a glycosylated product; and p30. Certain structural features of gp195 are shown in Figure 11, including: a hydrophobic tail at the C-terminus, indicating that the protein may be membrane anchored; clustered cysteine residues in the area of the C-terminus, suggesting significant disulfide bridging may be present; a hydrophobic leader sequence at the N-terminus; strain-variable tripeptide repeats in the area of the N-terminus; and a small region of tandem repeated amino acids with the sequence Glu-x or Thr-Glu-x in the middle of the protein.[38-40]

The organization of the processed products within Camp strain gp195 (Fig. 12B) was determined with antibodies affinity-purified from recombinant peptides expressed from overlapping pieces of the gene[36] (Fig. 12A). Antibodies selected from clones a23 and a50 reacted with p83, which was shown previously to be associated with the N-terminal end of gp195, and antibodies selected from clones a79 and a88 reacted with p83 as well as p45. Because these antibodies also reacted with 152 and 112 kDa processing intermediates, it was hypothesized that p83 and p45 were separated by a 30 kDa product that was not observed in our experiments. The assignment of gp45, which was identified with Mabs, to the C-terminal end of gp195 is consistent with the absence of glycosylation of the 152 and 112 kDa processing intermediates and the lack of reactivity of these Mabs with products and intermediates other than gp45.

Recently, Holder et al.[40] showed the existence of the putative 30 kDa product discussed above. Antibodies raised against the fusion proteins produced from plasmids containing overlapping pieces of DNA form the Wellcome strain gene (Fig. 12C) were used to precipitate merozoite surface antigens or probe immunoblots of parasite extracts. Although antibodies raised against the fusion proteins from constructs 9 and 10 reacted with a 28-30 kDa product, those raised against construct 1 did not.

Results of both these investigations indicate that the 30 kDa product that separates p83 from p45 normally is not immunogenic. Furthermore, although one may obtain antibody responses against nonimmunogenic regions of a protein by immunizing with peptides corresponding to that region, the choice of the peptide is an important consideration.

Malaria parasites can be divided into 2 groups based on the primary structure of the repeat re-
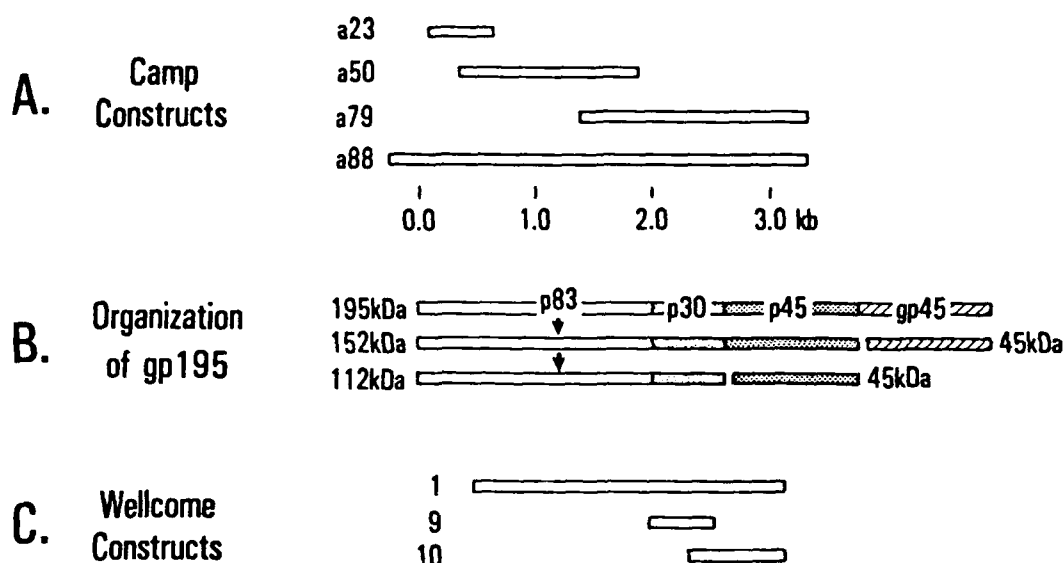
FIGURE 12. Organization of processed products deduced from reactivities of antibodies directed against recombinant constructs of the Camp and Wellcome strains of *P. falciparum*.

gions located near the N-terminus of gp195.[39] Examining the amino acid sequence of the repeat region from 7 parasite isolates reveals that the tandem repeated amino acid triplets are flanked by short sequences of amino acids that are conserved among groups of parasites (Fig. 13A), and the groups have been identified as Camp-like or Wellcome-like. Polyclonal antibodies and Mabs against these group-specific peptides should be useful in epidemiologic studies.

Our ability to raise antibodies against synthetic peptides corresponding to the conserved sequence at the C-terminal end of the repeats associated with Camp-like parasites (Fig. 13B)

depended on such variables as the primary structure of the peptide, the chemistry used for coupling the peptides to carrier, and the assay system used to evaluate the antibody reaction. Groups of 5 mice (C57BL/6) were immunized with a 24 amino acid peptide (24mer) or a 16 amino acid peptide (16mer) (Fig. 13B) coupled to keyhole limpet hemocyanin (KLH) with either glutaraldehyde (GLU) or carbodiimide (CDI). Antisera were evaluated by ELISA against noncoupled homologous peptide, and by IFA assay against the CAMP, FCR-3, and IMTM-22 parasite isolates. These isolates were chosen for the IFA assay studies because previous work with Mabs
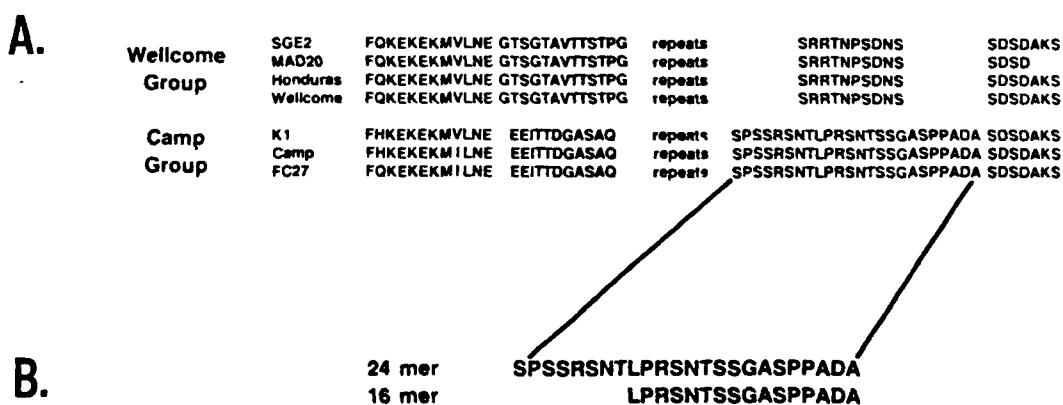

FIGURE 13. Comparison of regions flanking gp195 repeats in 7 *P. falciparum* isolates.

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TABLE 5

Results of immunizing mice with gp195 synthetic peptides coupled to KLH

Peptide	X-linker	ELISA	Immunofluorescence		
			Camp	FCR-3	IMTM-22
3'-Camp (24)	Glut	5/5	5/5	0/5	0/5
3'-Camp (16)	Glut	5/5	0/5	0/5	0/5
3'-Camp (24)	CDI	5/5	5/5	0/5	0/5
3'-Camp (16)	CDI	0/5	5/5	4/5	3/5

and DNA showed that this region of gp195 from the Camp strain is different from that of FCR-3 and IMTM-22 isolates.

Results of the analyses are shown (Table 5). Antibodies raised against the 24mer, whether coupled with glutaraldehyde (GLU-24) or carbodiimide (CDI-24), gave strong ELISA reactions against 24mer peptide, and reacted in IFA assay with Camp but not FCR-3 or IMTM-22 parasites. Antibodies raised against GLU-16 reacted with 16mer peptide in ELISA but did not react with parasites in IFA assay. However, antibodies raised against CDI-16 did not react in ELISA but reacted with all 3 parasite isolates in IFA. One explanation for this latter result is that coupling the peptide with carbodiimide created a new epitope by polymerizing the 16mer peptide. This epitope would not exist in the ELISA because polymerized antigen was not used as a substrate. The IFA results suggest that this putative epitope is present on a protein found in all 3 isolates. This interpretation is supported by the observation that antibodies against CDI-24 give a merozoite surface reaction that is typical for antibodies against gp195, whereas antibodies against CDI-16 give an amorphous reaction which is not specific for the merozoite surface (not shown). Although the 24mer and 16mer peptides are derived from the same region of gp195, only the 24mer was useful for producing specific antibodies against this antigen.

Recombinant and synthetic peptides are useful for studying host antibody responses to malaria parasite antigens. It is important to recognize, however, that peptides possess some of the characteristics associated with their protein counterparts. Significant among these are that some regions of a peptide may be more immunogenic than others and that antibodies raised against peptides may cross-react with unrelated antigens. Thus, the choice of peptide and the method

of presenting the peptide to the immune system may be critical factors to consider when applying peptide technology to areas of research such as vaccine development.

REFERENCES

1. Yoshida, M., Nussenzweig, R., Potocnjak, R., Nussenzweig, V., and Aikawa, M., 1980. Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasites. Science, 207: 71–73.

2. Cochrane, A. H., Santoro, F., Nussenzweig, V., Gwadz, R. W., and Nussenzweig, R., 1982. Monoclonal antibodies identify the protective antigens of sporozoites of Plasmodium knowlesi. Proc. Natl. Acad. Sci. USA, 79: 5651–5655.

3. Hollingdale, M. R., Nardin, E. H., Tharavanij, S., Schwartz, A. L., and Nussenzweig, R. S., 1984. Inhibition of entry of Plasmodium falciparum and P. vivax sporozoites into cultured cells; an in vitro assay of protective antibodies. J. Immunol., 132: 909–913.

4. Young, J. F., Hockmeyer, W. T., Gross, M., Ballou, W. R., Wirtz, R. A., Trosper, J. H., Beaudoin, R. L., Hollingdale, M. R., Miller, L. H., Diggs, C. L., and Rosenberg, M., 1985. Expression of Plasmodium falciparum circumsporozoite proteins in Escherichia coli for potential

use in a human malaria vaccine. *Science, 228:* 958–962.

5. Zavala, F., Tam, J. P., Cochrane, A. H., Quakyi, I., Nussenzweig, R. S., and Nussenzweig, V., 1985. Rationale for development of a synthetic vaccine against *Plasmodium falciparum. Science, 228:* 1436–1440.

6. Aley, S. B., Bates, M. D., Tam, J. P., and Hollingdale, M. R., 1986. Synthetic peptides from the circumsporozoite proteins of *Plasmodium falciparum* and *Plasmodium knowlesi* recognized the human hepatoma cell line HepG2-A16 in vitro. *J. Exp. Med., 164:* 1915–1922.

7. Dame, J. B., Williams, J. L., McCutchan, T. F., Weber, J. L., Wirtz, R. A., Hockmeyer, W. T., Maloy, W. L., Haynes, J. D., Schneider, I., Roberts, D., Sanders, G. S., Reddy, E. P., Diggs, C. L., and Miller, L. H., 1984. Structure of the gene encoding the immunodominant surface antigen on the sporozoite of the human malaria parasite *Plasmodium falciparum. Science, 225:* 593–599.

8. Godson, G. N., Ellis, J., Svec, P., Schlesinger, D. H., and Nussenzweig, V., 1983. Identification and chemical synthesis of a tandemly repeated immunogenic region of *Plasmodium knowlesi* circumsporozoite protein. *Nature, 305:* 29–33.

9. Vergara, U., Gwadz, R., Schlesinger, D., Nussenzweig, V., and Ferreira, A., 1985. Multiple nonrepeated epitopes on the circumsporozoite protein of *Plasmodium knowlesi. Mol. Biochem. Parasitol., 14:* 283–292.

10. Aley, S. B., Bates, M. D., Hockmeyer, W. T., Miller, L. H., and Hollingdale, M. R., 1986. Repeat region of *Plasmodium falciparum* circumsporozoite protein does not recognize or bind to human hepatoma target cell. *Mem. Inst. Oswaldo Cruz (Suppl. II), 81:* (In press).

11. Ballou, W. R., Rothbard, J., Wirtz, R. A., Gordon, D. M., Williams, J. S.,Gore, R. W., Schneider, I., Hollingdale, M. R., Beaudoin, R. L., Malloy, W. L., Miller, L. H., and Hockmeyer, W. T., 1985. Immunogenicity and immunologic activity of antibodies to synthetic peptides of the circumsporozoite protein of *Plasmodium falciparum. Science, 228:* 996–999.

12. Hoffman, S. L., Cannon, L. T., Sr., Berzofsky, J. A., Majarian, W. R., Young, J. F., Maloy, W. L., and Hockmeyer, W. T., 1987. *Plasmodium falciparum:* A T cell epitope on sporozoite vaccine allows sporozoite boosting of immunity. *Exp. Parasitol., 64:* 64–70.

13. Ballou, W. R., Hoffman, S. L., Sherwood, J. A., Hollingdale, M. R., Neva, F., Hockmeyer, W. T., Gordon, D. M., Wirtz, R. A., Young, J. F., Reeve, P., Diggs, C. L., and Chulay, J. D., 1987. Safety and efficacy of a recombinant DNA *Plasmodium falciparum* sporozoite vaccine. *Lancet, 1:* 1277–1281.

14. Arnot, D. E., Barnwell, J. W., Tam, J. P., Nussenzweig, V., Nussenzweig, R. S., and Enea, V., 1985. Circumsporozoite protein of *Plasmodium vivax:* Gene cloning and characterization of the immunodominant epitope. *Science, 230:* 815–818.

15. McCutchan, T. F., Lal, A. A., de la Cruz, V., Miller, L. H., Maloy, W. L., Charoenvit, Y., Beaudoin, R. L., Guerry, P., Wistar, R., Jr., Hoffman, S. L., Hockmeyer, W. T., Collins, W. E., and Wirth, D., 1985. Sequence of the immunodominant epitope for the surface protein on sporozoites of *Plasmodium vivax. Science, 230:* 1381–1383.

16. Barr, P. J., Gibson, H. L., Enea, V., Arnot, D. E., Hollingdale, M. R., and Nussenzweig, V., 1987. Expression in yeast of a *Plasmodium vivax* antigen of potential use in a human malaria vaccine. *J. Exp. Med., 165:* 1160–1171.

17. Del Giudice, G., Cooper, J. A., Merino, J., Verdini, A. S., Pessi, A., Togna, A. R., Engers, H. D., Corradin, G., and Lambert, P-H., 1986. The antibody responses in mice to carrier-free synthetic polymers of *Plasmodium falciparum* circumsporozoite repetitive epitope is I-A^b restricted: Possible implications for malaria vaccines. *J. Immunol., 137:* 2952–2955.

18. Good, M. G., Berzofsky, J. A., Maloy, W. L., Hayaski, Y., Fujii, N., Hockmeyer, W. T., and Miller, L. H., 1986. Genetic control of the immune response in mice to a *Plasmodium falciparum* sporozoite vaccine: Widespread non-responsiveness to single malaria T epitope in highly repetitive vaccine. *J. Exp. Med., 164:* 655–660.

19. Hancock, K., and Tsang, V. C. W., 1986. Development and optimization of the FAST-ELISA for detecting antibodies to *Schistosoma mansoni. J. Immunol. Meth., 92:* 167–176.

20. Favaloro, J. M., Coppel, R. L., Corcoran, L. I., Foote, S. J., Brown, G. V., Anders, R. F., and Kemp, D. J., 1986. Structure of the RESA gene of *Plasmodium falciparum. Nucleic Acids Res., 14:* 8265–8277.

21. Campbell, G. H., Brandling-Bennett, A. D., Roberts, J. M., Collins, F. H., Kaseje, D. C. O., Barber, A. M., and Turner, A., 1987. Detection of antibodies in human sera to the repeating epitope of the circumsporozoite protein of *Plasmodium falciparum* using the synthetic peptide (NANP)₃ in an enzyme-linked immunosorbent assay. *Am. J. Trop. Med. Hyg., 37:* 17–21.

22. Kilejian, A., 1979. Characterization of a protein correlated with the production of knob-like protrusions on membranes of erythrocytes infected with *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA, 76:* 4650–4653.

23. Leech, J. H., Barnwell, J. W., Aikawa, M., Miller, L. H., and Howard, R. J., 1984. *Plasmodium falciparum* malaria: Association of knobs on the surface of infected erythrocytes with a histidine-rich protein and the erythrocytoskeleton. *J. Cell Biol., 98:* 1256–1264.

24. Howard, R. J., Uni, S., Aikawa, M., Aley, S. B., Leech, J. H., Lew, A. M., Wellems, T. E., Rener, J., and Taylor, D. W., 1986. Secretion of a malarial histidine-rich protein (PfHRP-II) from *Plasmodium falciparum*-infected erythrocytes. *J. Cell Biol., 103:* 1269–1277.

25. Stahl, H. D., Kemp, D. J., Crewther, P. E., Scanlon, D. B., Woodrow, G., Brown, G. V., Bianco, A. E., Anders, R. F., and Coppel, R. L., 1985.

Sequence of a cDNA encoding a small polymorphic histidine- and alanine-rich protein from *Plasmouium falciparum. Nucleic Acids Res., 13:* 7837–7846.

26. Wellems, T. E.. and Howard, R. J., 1986. Homologous genes encode two distinct histidine-rich proteins in a cloned isolate of *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA, 83:* 6065–6069.

27. Kilejian, A., Sharma, Y. D., Karoui, H., and Naslund, L., 1986. Histidine-rich domain of the knob protein of the human malaria parasite *Plasmodium falciparum. Proc. Natl. Acad. Sci. USA, 83:* 7938–7941.

28. Wellems, T. E., Rock, E. P., Maloy, W. L., Taylor, D. W., and Howard, R. J., 1987. Histidine-rich proteins in *Plasmodium falciparum.* Pages 47–58 *in* N. Agabian, H. Goodman, and N. Noguier, eds., *Molecular Strategies of Parasitic Invasion,* UCLA Symposia in Molecular and Cell Biology, New series, Volume 42. Alan R. Liss, Inc., New York.

29. Holder, A. A., and Freeman, R. R., 1982. Biosynthesis and processing of a *Plasmodium falciparum* schizont antigen recognized by immune serum and a monoclonal antibody. *J. Exp. Med., 156:* 1528–1538.

30. Howard R. J., Lyon, J. A., Diggs, C. L., Haynes, J. D., Leech, J. H., Barnwell, J. W., Aley, S. B., Aikawa, M., and Miller, L. H., 1984. Localization of the major *Plasmodium falciparum* glycoprotein on the surface of mature intraerythrocytic trophozoites and schizonts. *Mol. Biochem. Parasitol., 11:* 349–362.

31. Holder, A. A., and Freeman, R. R., 1984. The three major antigens on the surface of *Plasmodium falciparum* merozoites are derived from a single high molecular weight precursor. *J. Exp. Med., 160:* 624–629.

32. Hall, R., Osland, A., Hyde, J. E., Simmons, D. L., Hope, I. A., and Scaife, J. G., 1984. Processing, polymorphism, and biological significance of p190, a major surface antigen of the erythrocytic forms of *Plasmodium falciparum. Mol. Biochem. Parasitol., 11:* 61–80.

33. McBride, J. S., Newbold, C. I., and Anand, R., 1985. Polymorphism of a high molecular weight

schizont antigen of the human malaria parasite *Plasmodium falciparum. J. Exp. Med., 161:* 160–180.

34. Pirson, P. J., and Perkins, M. E., 1985. Characterization with monoclonal antibodies of a surface antigen of *Plasmodium falciparum* merozoites. *J. Immunol., 134:* 1946–1951.

35. Howard, R. F., Stanley, H. A., Campbell, G. H., Langreth, S. G., and Reese, R. T., 1985. Two *Plasmodium falciparum* merozoite surface peptides share epitopes with a single M, 195,000 parasite glycoprotein. *Mol. Biochem. Parasitol., 17:* 61–77.

36. Lyon, J. A., Geller, R. H., Haynes, J. D., Chulay, J. D., and Weber, J. L., 1986. Epitope map and processing scheme for the 195,000 dalton surface glycoprotein of *Plasmodium falciparum* merozoites deduced from cloned overlapping segments of the gene. *Proc. Natl. Acad. Sci. USA, 83:* 2989–2993.

37. Lyon, J. A., Haynes, J. D., Diggs, C. L., Chulay, J. D., Haidaris, C. G., and Pratt-Rossiter, J., 1987. Monoclonal antibody characterization of the 195-kilodalton major surface glycoprotein of *Plasmodium falciparum* malaria schizonts and merozoites: Identification of additional processed products and a serotype-restricted repetitive epitope. *J. Immunol., 138:* 895–901.

38. Holder, A. A., Lockyer, M. J., Odink, K. G., Sanhu, J. S., Riveros-Morano, V., Nicholls, S. C., Hillman, Y., Davey, L. S., Tizard, M. L. V., Schwartz, R. T., and Freeman, R. R., 1985. Primary structure of the precursor to the three major merozoite surface antigens of *Plasmodium falciparum* merozoites. *Nature (London), 317:* 270–273.

39. Weber, J. L., Leininger, W. M., and Lyon, J. A., 1986. Variation in the gene encoding a major merozoite surface antigen of the human malaria parasite *Plasmodium falciparum. Nucleic Acids Res., 14:* 3311–3323.

40. Holder, A. A., Sandhu, J. S., Hillman, Y., Davey, L. S., Nicholls, S. C., Cooper, H., and Lockyer, M. J., 1987. Processing of the precursor to the major merozoite surface antigens of *Plasmodium falciparum. Parasitology, 94:* 199–208.